ZOOLOGICAL SCIENCE 8: 81–88 (1991)

Oestradiol-17β Affects differentially Viability, Progesterone Secretion, and Apical Surface Morphology of Hamster Ovarian Follicles *in vitro*

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ABSTRACT—Previous studies have shown that oestradiol-17β (OE_2) exerts profound stimulatory effects on rat granulosa cells (GC) *in vivo* and *in vitro* while exerting an atretogenic effect on ovarian follicles of monkeys. We wished to determine the effects of OE_2 on a model intermediate between *in-vivo* animal and *in-vitro* cell studies, that of explanted hamster follicles *in vitro*. Hamsters were sacrificed on the morning of proestrus, ovaries were removed, and preovulatory follicles were excised and placed in culture in the presence or absence of OE_2. Following culture, GC and oocyte viability were assessed. Additionally, culture media were collected at 24-hr intervals and analyzed for progesterone (P). Follicles remained viable by most indices. There was a slight increase in GC viability at 72 hr with 1 μg OE_2/ml. P accumulation was likewise transiently increased in the treated group at only 24 hr. Scanning electron microscopy, however, revealed that OE_2 treatment dramatically altered surface epithelial cells by increasing blebbing. The present study suggests that OE_2, at best, exerts only mildly stimulatory effects on viability and steroidogenesis of whole hamster follicles *in vitro*; this is comparable to effects seen in hamster GC, and *in vivo*, but is in stark contrast to the exaggerated stimulatory responses observed for the rat. Major changes in epithelial cell surface may designate this as a locus of OE_2 effects. We expect that this model will serve as a more physiologic paradigm than other *in-vitro* systems in the analysis of direct ovarian effects of estrogens and other bioactive molecules.

INTRODUCTION

We have in recent studies shown that oestrogen (OE) exerts a direct effect on ovarian cells. *In-vitro* culture of monkey granulosa cells (GC) showed that oestradiol-17β (OE_2) can reduce progesterone (P) output compared with untreated controls [1]. Similarly, diethylstilboestrol decreased OE output by hamster GC [2]. These and other data have contributed to the suggestion that OE from the dominant follicle (DF) may serve a physiologic role in follicle selection by acting to suppress the growth and function of additional follicles in the monkey [3, 4]. Such inhibitory effects of OE on follicle viability and steroid output in most species is in stark contrast to the augmentative effects that characterize rat GC *in vitro* [5–7].

To further investigate the effects of OE in a model intermediate between the rat and primate, and one that may be extrapolatable to the latter, we wished to culture hamster follicles long term *in vitro,* as has been demonstrated short term for the rat [8, 9]. This model will provide an efficient and more physiologic test system in which to study atresia other than that of monolayer culture of GC. This model will also allow us to bridge the gap between *in vivo* animal studies and in-vitro culture of GC with regard to a direct effect of OE_2.

MATERIALS AND METHODS

Animals

Mature (3–5 months of age) golden Syrian hamsters (*Mesocricetus auratus*) exhibiting at least

Accepted June 19, 1990
Received May 8, 1990
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two normal, 4- day cycles were weighed (156 g±5 [30]; $\bar{x}+1$ S.E.M. [n]) and sacrificed under ether anesthesia on the morning of proestrus. Trunk blood was collected, allowed to clot, and centrifuged ($500\times g$, 10 min) to obtain serum. Following sacrifice, ovaries were excised from the animal, cleaned of fat, and prepared for dissection. The largest preovulatory folicles (>500 μm diameter) were excised under a dissecting microscope equipped with a micrometer reticle.

Culture in vitro, *histology and oxygen consumption*

Follicles were placed in Falcon organ culture dishes (VWR, Chicago, IL) and cultured with the following constituents: Dulbecco's modified Eagle's medium (DMEM) mixed 1:1 with Ham's F-12 (Gibco, Grand Island, NY) supplemented with heparin (1 U/ml); gentamycin (100 ng/ml); HEPES buffer (12.5 mM); hFSH (100 ng/ml); hamster serum (10%; sterilized with a Millipore filter and extracted with dextran-coated charcoal); and in the presence or absence of E_2 (0.01, 0.1 or 1.0 μg/ml). Total culture volume measured 1.0 ml. Cultures were maintained for 72 hr in 5% CO_2 in air at 37°C.

Upon culture termination, GC and the oocyte were expressed from the follicle and suspended in approximately 50 μl of DMEM-F12. GC viability was assessed by direct observation of exclusion of 0.2% trypan blue by approximately 200 cells, and expressed as percent of control. Oocyte viability was assessed by direct observation of bright fluorescence using 6 μM fluorescein diacetate [10]. Brightly fluorescing oocytes were characterized as "+" and viable (due to liberation of fluorescein by intracellular non-specific esterases), and degenerate oocytes did not fluoresce (−). A Zeiss compound microscope equipped with epiflourescence (BT-exciter filter [450–490 nm], dichroic splitter filter [510 nm], and long-pass barrier filter [520 nm], Eberhardt Instr. Co., Downer's Grove, IL) was utilized in the above observations.

Three follicles from each group were retained for histologic study following culture. Follicles were fixed in Bouin's fluid for 72 hr immediately following culture termination, and subsequently placed in 70% ethanol until processed. Follicles were double embedded in agar (0.7 and 1.3%) [11], dehydrated in a graded ethanol series, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin. Sectioned material was analyzed for the number of GC per 1600 μm^2 area in the largest cross sections, taking a mean of 5 determinations per follicle.

A minimum of three follicles (range, 3–6) from control, OE_2-treated, and non-cultured (immediate, or "time-zero") groups were fixed overnight in 3% glutaraldehyde in Millonig's phosphate buffer (MPB) [12] at 4°C; washed twice in MPB for 10 min each; post-fixed in 1% osmium tetroxide in MPB, 25°C for 30 min; and washed again in MPB. Follicles were dehydrated in a graded ethanol series, and critical-point dried. Specimens were mounted and sputter-coated with gold at a thickness of 16 nm. SEM was performed with a Hitachi SEM Model S-570 using an accelerating voltage of 15 kV and a working distance of 10 mm. Polaroid 665 film was used for all photographs.

Follicular oxygen consumption ($\dot{V}O_2$) was measured polarographically with a Clark-type oxygen electrode with micromodifications (Yellow Spring, OH) [13]. Follicles (n=5–13/animal) from cycling hamsters were incubated either immediately or after the 72-hr culture period with or without OE_2 in 2 ml DMEM-F12 (pre-equilibrated in 5% CO_2 in air overnight and pre-stabilized for 2 hr) with continuous agitation at 37±0.01°C (Haake Model FE2 circulating water bath, Karlsruhe, FRG). $\dot{V}O_2$ by follicles was calculated from the observed decrease in O_2 tension (μL), (recorded at 30-min intervals), per unit time, minus $\dot{V}O_2$ by medium without follicles in a control chamber (0.17±0.01 [19] μL/h; $\bar{x}\pm 1$ S.E.M. [n]). $\dot{V}O_2$ was normalized per 10 follicles. A positive control was the addition of 1.5×10^{-2} M KCN, which reduced $\dot{V}O_2$ to baseline levels.

Radioimmunoassay

Culture medium (1.0 ml) was drawn off and resupplemented (1.0 ml) at 24-hr intervals. Aliquots were frozen and later analyzed for P using kits (Rapid Assay, Diagnostic Products Corp., Los Angeles, CA) and a Packard PRIAS CGD autogamma spectrometer (courtesy of Dr. J. Buntin). The human P assay kits were validated for the hamster by demonstrating parallelism between

samples in serial dilutions vs. the standard curve. Interassay and intrassay variation meausred 15.7± 3.9% and 9.7±1.7%, respectively. Sensitivity of this assay was 0.1 ng/ml. Accumulation of P was expressed as ng/ml of culture medium, normalized per 5 follicles.

Statistical analyses

For viability and endocrine studies, the follicles from one ovary were randomly assigned to the treated condition (OE_2), while the follicles from the contralateral ovary served as the control (no OE_2). Oocyte viability was compared using Chi-square or Fischer exact-probability test. A comparison of GC viability between experimental and control cultures was completed utilizing a paired t-test or Wilcoxon's signed-rank test.

Simple linear regression analyses were done to validate $\dot{V}O_2$ data; $\dot{V}O_2$ was linear over the first 2 hr. Comparisons of $\dot{V}O_2$ among groups were made by one-way analysis of variance (ANOVA). Assay data for P between pairs of ovaries were also analyzed by a paried t-test; while an analysis of P accumulation over time and treatment was performed using a two-way ANOVA ("stats" program, Dr. S. Sholl, Wisconsin Regional Primate Research Center), followed by a one-way ANOVA (upon significance) and a Student-Newman-Keuls test for multiple comparisons. $P<0.05$ was considered to be significant.

RESULTS

Preliminary validation of model

Long-term culture (72 hr) reduced oocyte viability (as assessed by fluorescence with FDA) to 87.6% (85/97), as compared with 100% (28/28) for oocytes recovered within 15 min of necropsy ($p<0.05$); and reduced GC viability from 46.1± 1.2% (7) ($\bar{x}\pm 1$ S.E.M. [n replicates]) to 40.7± 1.1% (10) ($p<0.05$). $\dot{V}O_2$ was the same for

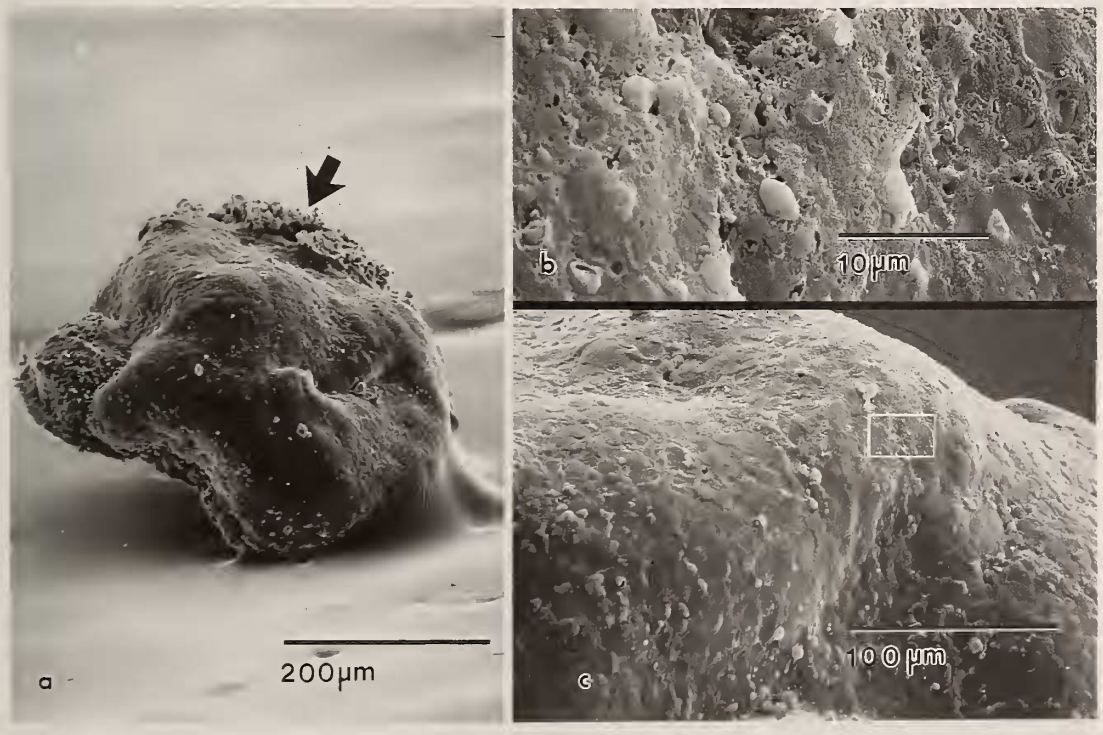

FIG. 1. Scanning electron micrographs of a preovulatory hamster follicle incubated for 72 hr in control medium (a-c). Note the smooth appearance of the external follicular surface, with some ruffled areas of theca present only in pathces (arrow) (a; magnification, ×130). At higher magnifications, surface epithelium appears squamous in nature (b×250; c×2500).

time-zero controls as for 3- day cultures (1.31± 0.18 [10 replicates] μl O_2/hr/10 follicles vs. 2.17± 0.38 [4], respectively).

Follicles remained steroidogenically active in culture as evident from their similar steroid output during each of three consecutive, 24-hr periods (5.55±1.40 [8] ng P/ml/ 5 follicles, 24 hr; 8.69± 0.90 [8], 48 hr; 8.90±0.50 [8], 72 hr; $p>0.05$). Preliminary observations with transmission electron microscopy (TEM) showed that the basal lamina remained intact throughout culture. Light microscopy showed that the follicles and oocyte appeared normal in histologic section, with no overt signs of atresia.

Present study

The inclusion of OE_2 in the culture medium had no effect on oocyte viability as determined with FDA (87.6% [85/97], − OE_2; 85.7% [30/35], + 100 ng OE_2/ml; 88.7% [47/53], +1 μg OE_2/ml; $p>0.05$). A small but significant increase in the percentage of viable GC was observed for follicles cultured in the presence of 1 μg OE_2/ml compared with paired controls (42.4±0.9% [10] vs. 40.7± 1.1% [10]; $p<0.05$ by paired t-test); 10- or 100-fold lower concentrations OE_2 exerted no effect in this regard. There was no change in the apparent GC density with treatment (19.5±2.8 [3 replicates, each a mean of 5 random determinations] GC/1600 μm^2, − OE_2; 16.7±1.3 [3], +1 μg OE_2/ml).

OE_2 did not affect $\dot{V}O_2$ during either short-term (2 hr: 1.18±0.1 [15 replicates] μl O_2/hr/10 follicles, − OE_2 vs. 1.76±0.40 [5], + OE_2), or long-term culture (72 hr: 2.02±0.49 [3], − OE_2 vs. 1.84±0.20 [5], + OE_2; $p>0.05$).

The ovarian surface epithelium overlying the apical region (that exposed site on the follicluar surface where ovulation will presumably occur) of preovulatory follicles fixed immediately (time-0 controls) or incubated 3 days in medium alone, appeared squamous in nature, as revealed by

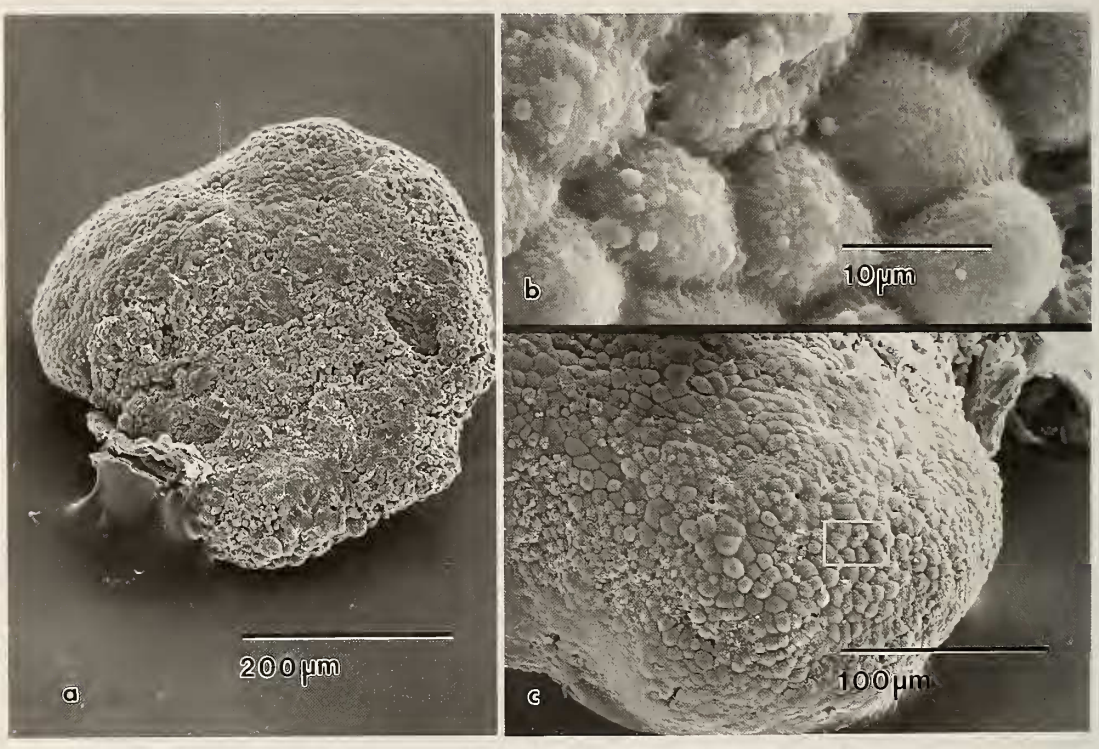

Fig. 2. scanning electron micrographs of a preovulatory hamster follicle incubated for 72 hr in medium containing 1 μg OE_2/ml (a-c). Note the prevalence of rounded, columnar surface epithelium and of blebbing on most of the apical surface (a×130; b×250, and c×2500 are from a second, treated follicle).

TABLE 1. Effect of oestradiol-17β (OE_2) on accumulation of progesterone (P) by explanted follicles in culture[1]

	24 hr	48 hr	72 hr	Total
Control	8.29±2.83[2] (8)	16.31±5.78 (9)	11.36±3.16 (10)	35.96±3.92 (27)
1 μg OE_2/ml	15.05±7.02* (9)	11.86±2.48 (10)	22.25±9.84 (10)	49.16±6.50 (29)

[1] Note that although a paired paradigm was utilized, numbers are presented as unpaired, parametric data for ease in representation.
[2] Accumulation of P over each 24-hr period or over the entire 72 jr ($\bar{x}$ ng P/ml±1 S.E.M.), normalized per 5 follicles. Number in parentheses denotes replications.
* Statistically different from controls using a Wilcoxon's signed-rank test (P=0.05).

scanning electron microscopy (SEM) (Fig. 1a); this was substantiated by higher magnifications of the same follicle (Fig. 1b, c). The ruffled, jagged areas observed in Figure 1 (primarily thecal tissue) maintained their sharp contours at higher magnifications (not shown). Figure 2a depicts the marked changes in the entire apical surface with OE_2 treatment, as it was now comprised of hemispherical columnar or cuboidal epithelial cells with numerous blebs over their surfaces (Fig. 2b, c). In certain areas, thecal tissue exhibited similar, OE_2-induced alterations (not shown).

Table 1 compares the mean P accumulation by treated (1 μg OE_2/ml) and control cultures at 24, 48 and 72 hr of culture. There was a slight, but significant, increase in P accumulation by treated follicles over controls only at 24 hr as determined by paired statistical analysis. This augmentation of P accumulation was abolished by 48 and 72 hr; similarly, there was no difference in total P accumulation over 72 hr between control and experimental groups. Treatment of follicles with 10 or 100 ng OE_2/ml was ineffective in altering P accumulation (data not shown).

DISCUSSION

The intent of the present study was to characterise a novel model for the study of direct effects of OE_2 at the level of the ovary by using intact preovulatory follicles from normally cycling hamsters, and correlating, uniquely, topographic changes with follicle viability and steroidogenesis. We observed no loss of steroidogenic capacity over the duration of culture, suggesting that follicles remained viable for 72 hr; these data correlated with their normal appearance in histologic section and with the lack of a difference in oxygen consumption seen between follicles analyzed immediately and those cultured for 72 hr [14]. There were reductions in oocyte and GC viability with culture, but these indices remained within the normal range [2]. Collectively, these data served to validate this long-term culture system as a model to investigate the effects of various bioactive molecules on follicles *in vitro*. Secondly, this study has shown that OE_2, at a concentration of 1 μg/ml (i.e., physiologic with respect to concentrations in follicular fluid, [15]), did little to alter the apparent viability of GC from hamster follicles *in vitro*.

Although OE_2 minimally altered GC viability in cultured follicles, it appeared to exert a more profound effect on the follicular surface, as SEM revealed increased epithelial and some thecal blebbing of the treated follicles; (TEM studies are underway to characterize these blebs). We have previously shown that, *in vivo,* the follicular effects of E_2 are specific, as cholesterol had no effect [16, 17]. Preliminary studies with SEM using an OE_2-receptor antagonist, CI-628, showed an obliteration of the OE_2-induced effects on epithelial morphology. Regarding theca, this layer is an important steroidogenic component of the follicle, and may be a site of initiation of follicular processes such as atresia [18]. It is interesting to note, however, that the more drastic ultrastructural changes in the OE_2-treated follicle as revealed by SEM were in the apical epithelium, and estrogen receptors in the monkey are by immunocytochemistry primarily localized in the ovarian germinal epithelium [19]. Although OE_2 receptors are present in hamster follicles, their distribution has not been established [20]. OE_2 treatment in-

creased blebbing of the follicular apical surface, identical to that shown for hCG-induced preovulatory changes in the hamster follicle *in vivo* by Pendergrass and Reber [21]; OE_2 may therefore mediate the effects of hCG observed by these workers, although interactions of OE_2 and hFSH/serum in producing the observed effects cannot be excluded from the present study. Certainly, enhanced surface area (blebbing) was not observed either in time-0 or 3- day control follicles, and was attenuated in the presence of OE_2 without serum (Hutz, preliminary observations); the former conditions were similar to those described *in vivo* for the untreated state [22]. Increased blebbing and microvillous projections have also been shown for cumulus cells isolated after the gonadotropin surge [23]; for GC taken from preovulatory follicles of rats primed with OE_2 and FSH and subsequently injected with hCG [24]; and for post-ovulatory cumulus cells [25]. Since in the present study, follicles were explanted on the morning of proestrus, substantially prior to the gonadotropin surge, the effect we observed was certainly due to OE_2 treatment. Functionally, the blebbing and other cytoplasmic projections appear to correlate with mucification (we have noted accumulation of hyaluornidase-sensitive material in follicles of OE_2-treated monkeys [26], with increased cell surface area, and with density of hCG-receptors [27, 28]. The injection of E_2 to hypophysectomised rats augmented the junctional surface area (gap junctions) of GC within 48 hr [29] and exerted similar effects on theca [30]. These gap junctions are apparently important in spreading hormonal signals among granulosa and theca cells by improving cell-cell intercommunication [31].

Treatment of hamster follicles with 1 μg OE_2/ml of culture medium produced a slight, transitory increase in P accumulation at 24 hr of incubation; this increase was, however, abolished at 48 and 72 hr. These results correlate with similar observations by Hutz *et al.* [2] in which incubation of dispersed hamster GC with OE_2 (100 ng/ml) and FSH augmented P accumulation over that of controls. The same study, however, showed OE_2 to be ineffective in the absence of FSH, suggesting that OE_2 synergized with FSH to augment P output. These data may reveal a direct, transitory effect of OE_2 early in the steroidogenic pathway, (e.g., enhancing activity of 3β-HSD), thereby leading to increased P synthesis [32]. Alternatively, OE_2 may function to briefly inhibit enzyme pathways beyond P (e.g., 17α-hydroxylase), leading to P accumulation [33].

It is not known whether the alterations in cell morphology in the present study are related to augmented P accumulation, since the increase in P was localized at 24 hr, and differences in surface morphology were not evident until 72 hr of culture. Regardless, the effects of OE_2 on long-term hamster follicle steroidogenesis and viability in this system appeared to be brief and minimal. The endocrinologic events in this hamster model therefore appeared to relate more closely to those in higher species than did events in the rat; for example, several studies have shown that OE-treated rat GC exhibited many-fold increases in steroid production [5, 34], while OE exerted negative effects in domestic species and other taxa, including primates [7, 26, 35, 36]; a folliculolytic effect or reduced sensitivity to E in the hamster *in vivo* [37, 38]; mild or non-stimulatory effects on hamster GC *in vitro* [2, 39]; and atretogenic effects of OE_2 in the monkey both *in vivo* [16] and *in vitro* [1]. The rat, in contrast, appears to be quite unique in its exaggerated positive response to OE both *in vivo* and *in vitro* [6, 40].

Results of the present study may be enumerated as follows: (1) The morphologic, biochemical, and endocrine data validate our model system of long-term follicle culture and indicate that OE_2 exerts at best only a mildly positive effect on granulosa cell viability and steroidogenesis, with no apparent effect on cell density. (2) OE_2 treatment markedly alters surface epithelium characteristics, therefore implicating this site as a possible locus for estrogen's effects on the ovarian follicle. (3) The minimal effect OE_2 exerts on hamster follicle steroidogenesis *in vitro* is in stark contrast to the markedly augmented effects characteristic of rat granulosa cells. Collectively, the results of the present study suggest that explanted hamster follicles provide an appropriate model with which to study the effects of OE_2 and other bioactive molecules at the level of the ovary. These studies may be more physiologically relevant to events occur-

ring in higher species (e.g., primates) than in rats.

ACKNOWLEDGMENTS

The authors wish to thank Brian Kiedrowski and Robert Zachow for technical assistance with the SEM graphics, Cheryl Fischer for assistance with the histology and radioimmunoassays, Leslie Quandt and Susie Hussong for help with $\dot{V}O_2$ determinations, Blong Sayaovang for light microscopic follicle analysis, Dr. S. Raiti for generously providing the hFSH, and Alnita Allen and Dorothy Perry for the faithful preparation of this manuscript. This work was supported by the UWM Graduate School and School of Letters and Sciences, a Sigma Xi Grant-in-aid of research to S. M. B., and an Endocrine Society Fellowship to P. K. Portions of this report were presented at the Mini-symposium on Reproductive Biology, Evanston, IL, USA, October 26, 1987, and at the Annual Meeting of the Endocrine Society, Seattle, WA, USA, June 22, 1989.

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